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THE "REACTION" OF BACTERIOLOGIC CULTURE MEDIA *

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INTRODUCTION

It would be a large task to compile all the recorded instances in which the reaction of a medium has influenced the morphology, pigment formation, proteolytic activity, rate of fermentation, or even the mere viability of bacteria. In 1895 Fuller[1] had collected "over a hundred" such cases; to-day they occur so frequently in the literature that there is no need to emphasize the importance of the subject.

Adjustments in the reaction of bacteriologic culture media are generally accomplished by titrations. General use of such methods dates from the suggestions made by Schultz[2] (1891), Fuller[1] (1895) and others in the early nineties. Founded upon these methods are the "scales of reaction." They are eminently useful, and, now that a vast deal of laboriously-gained experience has dictated in terms of these scales the proper reaction of important media, they are not likely to be easily displaced.

But the theory of titration has undergone a fundamental change since the origination of these methods, and we are now able to gain a clearer conception of many points which were of necessity inadequately treated by the early writers. Furthermore, the physiologic importance of "titratable acidity" is dwindling before the broader conceptions of modern physiologic and physical chemistry, and it may be seriously suggested that too strong an adherence to the old titration methods is a hindrance to progress.

It has long been recognized that acidity and alkalinity are best defined in terms of hydrogen ion concentration. Such a conception certainly correlates a vast number of facts. The rate of enzyme action, the stability of colloidal structures upon which cellular life depends, the solubility of many physiologically important compounds,

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* Received for publication May 3, 1915. Published by permission of the Secretary of Agriculture.

[1] Jour. Am. Pub. Health Assn., 1895, 20, p. 381.

[2] Centralbl. f. Backteriol., I, O., 1891, 10, p. 52.

as well as the structure and composition of media and the color of the indicators used in the adjustment of their reactions—all are dependent in greater or less measure upon hydrogen ion concentrations.

This is doubtless very generally recognized; but no attempt has been made, so far as the writer knows, to apply these principles extensively to bacteriologic culture media.

Hildebrand,[3] in assembling some of the principles of titration for use by chemists, has suggested that the application of such principles may have been delayed because the apparatus or the mathematics involved have appeared too complicated for men not trained in theoretical chemistry. Some of the difficulties involved in older methods may be avoided by the use of the hydrogen electrode. With this instrument we may determine directly hydrogen ion concentrations. Important points in the data so obtained may then be presented by graphic methods quite as well as by equations. In order to make these presentations clear, it will be necessary to sketch certain principles and to deal with certain questions which already have been set forth more adequately by others. To Hildebrand's[3] paper and to Michaelis'[4] monograph, the writer is particularly indebted.

METHODS

It may be well to recall that the customary method of determining hydrogen ion concentrations electrometrically depends upon a measurement of the potential of the chain:

Hg HgCl/n/10 KCl/saturated KCl/solution/PtH_2, for which we have at 30 C. the equation $E - 0.337 = 0.0601 \log 1/CH^+$, in which E is the total E. M. F. of the chain, 0.337 is the potential of the "tenth normal" calomel electrode, in terms of "the normal hydrogen electrode," and CH^+ is the hydrogen ion concentration of the solution. This equation may be transposed to $E - 0.337/0.0601 = \log 1/CH^+$. The value of $\log 1/CH^+$ or $- \log CH^+$ is obtained directly, and since it is a much more convenient value to plot than the unwieldy value of the corresponding hydrogen ion concentration, it is used under the symbol pH^+ introduced by Sørensen.[5]

In the experiments to be described later, this chain was used at 30 C. The electrode vessel was a modification of Hasselbalch's[6] and will be described in detail elsewhere. It was found that the contact potential between the n/10 KCl and the solution was reduced to a minimum by the use of the customary inter posed solution of saturated KCl. The design of the apparatus was such that this could be accomplished especially well, and since the corrections by Bjerrum's[7] method of extrapolation would be small and would have little effect upon relative values, they were neglected.

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[3] Jour. Am. Chem. Soc., 1913, 35, p. 847.

[4] Das Wasserstoffionen-Konzentration, 1914.

[5] Compt. rend. du Lab. Carlsberg, 1909, 8, p. 1.

[6] Biochem. Ztschr., 1913, 49, p. 451.

A small correction for barometric pressure and sometimes a correction of the calomel electrode, found by comparison with other assumed standards, were applied.

The electrodes were platinized platinum or gold coated with palladium black. The two types agree excellently, and no electrode was used which differed from the five comparison electrodes by more than 0.0001 volt after a night in the same solution. In the great majority of measurements, the electrodes used had agreed within ± 0.000,030 volt before they were transferred to the electrode vessel.

All solutions were brought to 30 C. ± 0.1° in a water bath and then, before measurement, their containers were wiped dry and they were left about half an hour in the air thermostat containing the hydrogen electrode. This thermostat was kept constant to within ± 0.05 C. (Beckmann thermometer reading) by a special regulator.[8] Aside from a slow drift in temperature, the fluctuations from moment to moment were less than ± 0.003 °, determined by an eight-pair copper constantine thermo element.

Measurements of potential were made with a Leeds and Northrup potentiometer and a galvanometer as null point instrument. The potentiometer was calibrated by the Bureau of Standards. The known potential was derived from a Weston cell, frequently compared with two Weston cells which had been standardized and loaned by the Bureau of Standards.

The sodium hydroxid used in titrations was practically carbonate free. It was made from carefully cleaned Kahlbaum sodium held in a silver crucible. The crucible was placed in a desiccator containing a layer of distilled water and held there until the sodium was completely converted to NaOH. From this the standard solution was made up. It was held in a paraffined Jena flask with protecting soda lime tubes and was delivered to a measuring burette through a glass tube sealed to the burette. The standardization was made by Morey's[9] benzoic acid method.

In cases in which lactic acid was used in titrations, the acid was boiled in dilute solution to convert the anhydrid to acid.

In stating the composition of media, the percentages of peptone, gelatin, etc., are not based on dry weights, since the object was to present data on media as ordinarily made up. Attention should also be drawn to the fact that a "1 per-cent peptone" solution is a solution made by treating 1 gm. Witte peptone with 100 c.c. water, heating, filtering, and making up to volume. By no means all the peptone goes into solution.

METHOD OF PRESENTING DATA

In presenting results, use is to be made of titration curves and it is essential that these should be understood. As an illustration, let us borrow an example given by Böttjer[10] and reproduced by Hildebrand[3] and Bjerrum.[11] In Chart 1 are sketched the titration curves of hydrochloric and acetic acids.

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[7] Ztschr. f. Electrochem., 1911, 17, p. 389.

[8] Jour. Am. Chem. Soc., 1913, 35, p. 1889.

[9] U. S. Dept. Commerce and Labor, Bureau of Standards, 1913, Bull. 8, p. 643.

[10] Ztschr. f. physik. Chem., 1897, 24, p. 253.

Let us start with 10 c.c. of n/10 HCl. Approximately 90 percent of the acid is dissociated. Hence, the normality of hydrogen ions in this 0.1-N HCl solution is about 0.09, written $CH^+ = 9.0 \times 10^{-2}$. If to this solution successive portions of n/10 NaOH are added, the acid becomes neutralized and the hydrogen ion concentration declines. After each successive addition of NaOH, we may measure the hydrogen ion concentration with a hydrogen electrode. In this measurement we obtain directly, as shown under Methods, the value of log $1/CH^+$, which, under the symbol pH^+, we shall use in plotting the results. It will be noticed that pH^+ increases as CH^+ decreases. All the charts in this paper have been constructed to show a rise in the curves with a rise of hydrogen ion concentration, and conversely, a fall in the curves with a decline in hydrogen ion concentration. On the abscissae of Chart 1, will be found the cubic centimeters of n/10 NaOH added to each 10 c.c. of titrated solution.

Plotting the results during a titration of HCl with NaOH, we obtain the curve shown in Chart 1. Each addition of NaOH causes a fall in hydrogen ion concentration, but it is not until almost all the acid has been neutralized that a sudden fall in the curve appears. When 99.9 percent of the acid has been neutralized, we should still have a 0.0001 N. HCl solution which, diluted with the water of the NaOH solution added, has become approximately 0.00005 N. HCl. Neglecting the influence of the NaCl and assuming that the acid is completely dissociated, we should then have a hydrogen ion concentration of approximately 5×10^{-5}. The next 0.07 c.c. of alkali added causes a precipitous drop in the hydrogen ion concentration, and, as the point of complete neutralization is passed, the curve falls rapidly to the region where we find the hydrogen ion concentrations of the sodium hydroxid.

It will be remembered of course that, according to the fundamental equation, Concentration $H^+ \times$ Concentration $OH^- =$ a constant, we must have hydrogen ions even in the presence of an excess of hydroxyl ions. Therefore, the alkalinity of solutions may still be defined in terms of the concentration of the hydrogen ions.

Turning again to Chart 1, we find there the "titration curve" of a weak acid, acetic. We proceed as before, and we notice in the first

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[11] Die Theorie der Alkalimetrischen und Azidimetrischen Titrierungen, 1914.

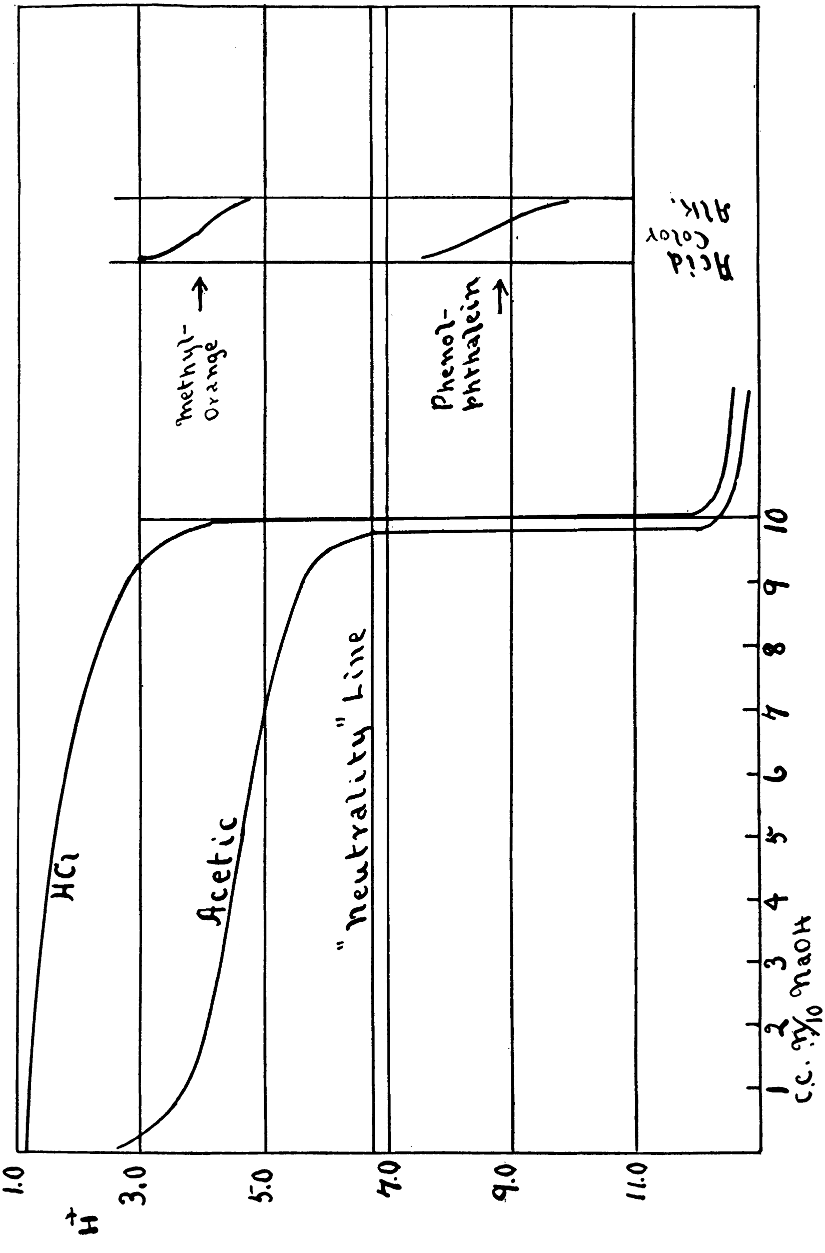

Chart 1.—Titration curves of hydrochloric and acetic acids.

place that the initial hydrogen ion concentration is much lower than that of the corresponding hydrochloric acid solution. In other words, acetic acid is weakly dissociated. Furthermore, the formation of sodium acetate upon the first addition of NaOH depresses the hydrogen ion concentration. Then the curve falls slowly until, as complete neutralization of the acid is approached, we have again the precipitous drop in the curve. All the while, however, the hydrolysis of the sodium acetate, as well as the effect of the acetate ion upon the dissociation of HAc, has made itself felt; and so, before complete neutralization is reached, the drop in the curve takes place. When the acid and alkali are present in equivalent concentration, the solution is distinctly alkaline, and we have, instead of a neutral salt, as in the case of NaCl, a hydrolyzed salt of a weak acid, with a strong base.

USE OF INDICATORS IN TITRATION

We may illustrate the usefulness of indicators in titrations by means of the titration curves just given. Without discussing the dissociation, tautomeral changes, or, in some cases, the colloidal properties of indicators, we may define them as substances which undergo changes in color within certain zones of hydrogen ion concentration. Through the work of Fredenthal,[12] Fels,[13] Salessky,[14] Salm,[15] Sørensen,[5, 16] Palitzsch,[17] Michaelis and Rona,[18] Walpole,[19, 20] and others, we now know the range of color changes of a large number of indicators. Phenolphthalein, for instance, to the unaided eye changes gradually from colorless to red between about $CH^+ = 1 \times 10^{-8}$ and 1×10^{-10}, while methyl orange undergoes its color change between about $CH^+ = 1 \times 10^{-3}$ and 4×10^{-5}.

Particular emphasis, as we shall see later, must be placed upon the fact that the color change occurs in a zone of appreciable width.

An attempt has been made to present graphically the zone of color change. The degree of color of phenolphthalein at any point between $pH^+ = 8.00$ and $pH^+ = 10.00$ is shown with an accuracy sufficient for present purposes by the curve of color change shown at the right in Chart 1, and reproduced in the other charts. In the same way, the curve of color change of methyl orange illustrates the passage of the color of this indicator from its full acid color over into its alkaline color as the hydrogen ion concentration falls from $pH^+ = 3.0$ to $pH^+ = 4.7$. The zones of color changes of these two indicators are actually much wider than the limits assigned here, but for all practicable purposes the limits assigned will do, since the zones given are those within which the eye detects differences in the color.

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[12] Ztschr. f. Electrochem., 1904, 10, p. 113.
[13] Ibid., p. 208.
[14] Ibid., p. 204.
[15] Ztschr. f. physik. Chem., 1906, 57, p. 471.
[16] Biochem. Ztschr., 1910, 24, p. 381.
[17] Ibid., 1911, 37, p. 131.
[18] Ztschr. f. Electrochem., 1908, 14, p. 251.
[19] Biochem. Jour., 1913, 7, p. 260.
[20] Ibid., 1914, 8, p. 628.

Turning again to the titration curve for HCl, we find that when the zone is reached in which methyl orange changes color, the curve has begun to drop, and before we are out of this zone, the curve is falling precipitously. When, therefore, practically every trace of HCl has been neutralized, methyl orange, if present, would show its full alkaline color. Furthermore, a minute addition of NaOH, not only sends the curve through the zone of methyl orange, but straight through a zone far below, in which phenolphthalein changes color. Consequently, both methyl orange and phenolphthalein will display a sudden color change in such a titration and we should describe both indicators as "sensitive."

On the other hand, if we should attempt to use methyl orange in titrating acetic acid, we should find no sudden sharp color change, or "end point." This is simply due to the fact that in titrating acetic acid, as the curve shows, the hydrogen ion concentration passes gradually through the zone of color change of methyl orange. If we wish to observe a sharp color change, we must seek an indicator whose transformation occurs in a region where the titration curve falls precipitously. It will be seen that an indicator suitable to the titration of acetic acid is phenolphthalein.

It does not always follow that a precipitous drop in the titration curve occurs when acid and alkali are present in equivalent amounts. This is shown to some extent by the acetic acid curve. Much more complicated situations arise in the titration of "very weak" acids and their mixtures. An exposition of the principles applicable under these circumstances is given by Bjerrum.[11]

In the titration of most media, containing as they do phosphate and various salts, protein, and amino-acids, often of unknown structure and generally in unknown relative proportions, it is impossible to decide to what point the mixture must be titrated in order to neutralize the acid groups. When such a mixture is titrated, the only thing accomplished is to reach some more or less definite hydrogen ion concentration as determined by the color of an indicator.

"BUFFER" EFFECT

There is one more point which we may illustrate by the curve for acetic acid. When we reach the region where both acetic acid and sodium acetate are present in appreciable quantities, we have a solution the hydrogen ion concentration of which is not easily altered by addition of either acid or alkali. This is expressed graphically in the flatness of the curve. If we add acid to an acetic acid-sodium acetate mixture, a portion of the sodium may be roughly described as parting company with the acetate ion to form a new salt, leaving the acetate ion to become weakly dissociated acetic acid. Largely because of this "weakness" in the dissociation of acetic acid, the hydrogen ion concentration of the mixture is not greatly affected. So, too, the addition of a small amount of alkali does not greatly depress the hydrogen ion concentration, since the alkali finds plenty of undissociated acetic acid with which to neutralize itself.

This is a very rough sketch of an example of what is known as "buffer" action. Any substance which tends to preserve the original hydrogen ion concentration of its solution upon the addition of an acid or a base, is called a "buffer" or "regulator."

TITRATION CURVES OF CULTURE MEDIA

In Charts 2-6 are shown the titration curves of some important media. In these figures the abscissae represent the amount of acid or alkali added (acid to the right, alkali to the left of the 0 ordinate). On the ordinate are to be found the pH^+ values. As a reference line, that of $pH^+ = 6.86$ is drawn to represent the hydrogen ion concentration of pure water at 30 C. This point is "absolute neutrality." The point $pH^+ = 8.50$ is assumed to be the "end point" of phenolphthalein, an arbitrary assumption, which will have to be used in later discussions. Accordingly, the $pH^+ = 8.50$ line has been made heavy to serve as another reference line.

To determine the values from which these titration curves were constructed, the following procedure was followed:

> Aliquot samples of a medium were apportioned in clean Jena Erlenmeyer flasks. To each was added a known amount of n/10 acid or alkali. These flasks were suspended in a water bath at 30 C. for about one-half hour; then having been wiped dry, they were placed in the 30 C. thermostat containing the hydrogen electrode. They were left there for another half hour. Then the determinations were made on each sample with care that, before each determination, the electrode vessel should have been thoroughly rinsed with a portion of the sample to be tested.

A study of the data so obtained reveals many interesting facts, which will be considered under separate headings.

REACTION OF CULTURE MEDIA TO PHENOLPHTHALEIN

Phenolphthalein is the indicator most commonly used in the titration of culture media. It is important therefore to note certain difficulties which attend its use.

It has already been pointed out that phenolphthalein, like all indicators, does not change color suddenly upon passing a certain hydrogen ion concentration. The zone of color change is broad, and it is only when a titration curve falls precipitously through this zone that the indicator flashes from one color to another.

With each of the titration curves (Charts 2-6), there will be found a graphic representation of the color change of phenolphthalein. This method of representation has been explained under Methods of Presenting Data. It readily will be seen that no sharp color change is to be expected in the titration of any of the media. In other words, none of the titration curves falls precipitously through the zone of phenolphthalein color change. Therefore, there can be no sharp end point in such a titration. To show the variation which arises from the assumption of different tints as end points, a 1 percent peptone solution and a 5 percent peptone solution were submitted to four bacteriologists and four chemists for titration. In each case the observer used the same calibrated burette and pipette, the same n/40 NaOH solution, the same phenolphthalein solution, the same carefully rinsed flasks, the same white background. Each observer, however, was asked to proceed as he was accustomed to do. The following results were obtained.

TABLE 1

TITRATION OF PEPTONE SOLUTIONS

Observer	Amt. in c.c. of n/40 Alkali Required to Each 5 c.c. of	
	1 Percent Peptone	5 Percent Peptone
Bacteriologist A	0.76	3.35
Bacteriologist B	0.80	3.25
Bacteriologist C	1.40	5.10
Bacteriologist D	1.00	4.45
Chemist E	0.58	2.68
Chemist F	1.12	7.40 (heated)
Chemist G	0.70	4.20
Chemist H	0.83	4.38

These wide differences are due, in part, to the variations in procedure and appear large because of the dilute alkali used; but for the most part the differences are attributable to the assumption of various tints as the end point. This will become strikingly evident if the reader will transpose the titers given above to the titration curves of the 1 percent and 5 percent peptone solutions. The locations will be found to fall along the curves within almost the entire range of color change of phenolphthalein.

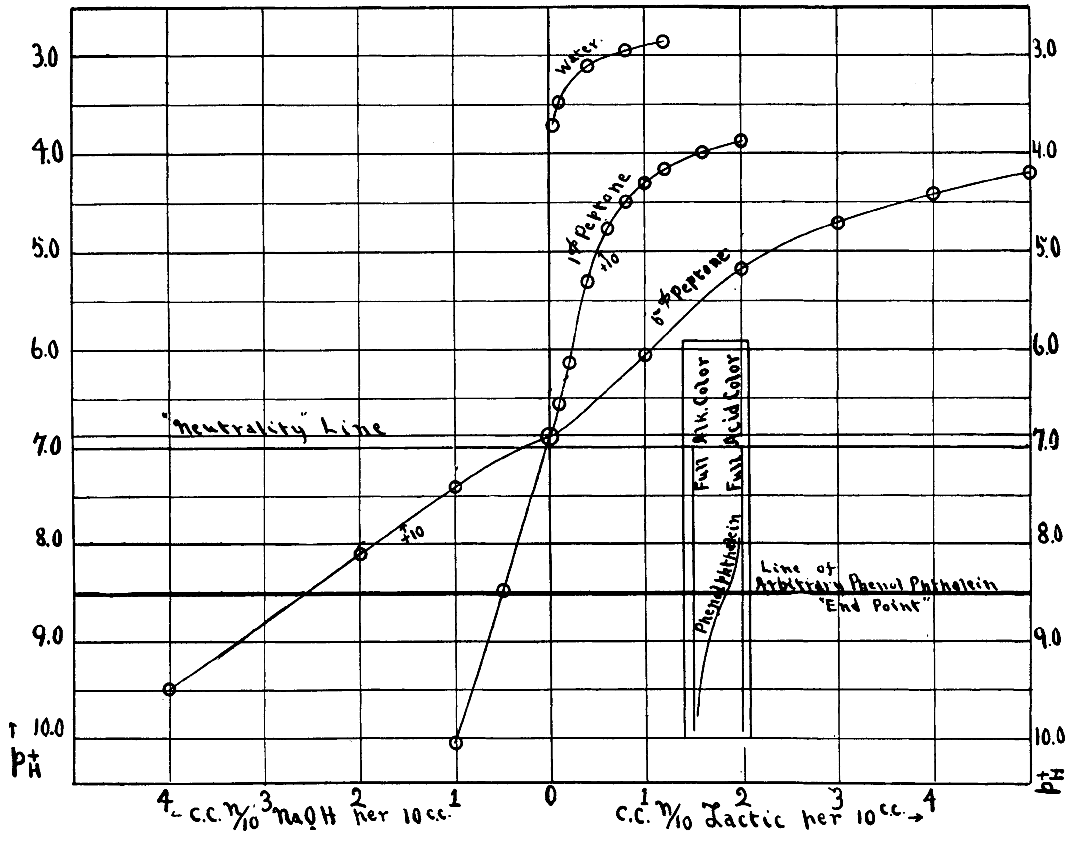

Chart 2.—Titration curves of 1 percent and 5 percent solutions of Witte peptone.

This recalls the well-known necessity for fixing some arbitrary tint as standard. Such a standard is defined by the American Public Health Association[21] as follows: "A faint but distinct pink color marks the true end point. This distinct pink color may be more precisely described as a combination of 25 percent red (wave length approximately 658) with 75 percent white, as shown by the disks of the color top, . . ."

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[21] Standard Methods for the Examination of Water and Sewage, 1912.

There are several objections to such a standard. In the first place, as Ridgeway[22] has emphasized, it is extremely difficult to reproduce lithographically a particular color. Furthermore, when produced, it fades upon exposure. Perhaps these objections would be of small consequence if comparison paper colors could be conveniently used. They certainly can not be so used. The most serious objection to such a standard is that no precise physical significance is attached to it, and without such a significance the standard may justly be ignored and is ignored.

A standard with an exact physical significance for the problem at hand would be a solution of known hydrogen ion concentration containing a definite amount of phenolphthalein. Solutions with well-defined hydrogen ion concentration may be accurately reproduced from acetic acid-sodium acetate mixtures,[23] phosphate mixtures,[24] and borate mixtures,[5] as well as from a variety of other mixtures.

In passing, it may again be emphasized that the titrimetric method has for its basis the color change of an indicator within a zone of hydrogen ion concentration, and that the sharpness with which the color change appears is dependent upon the slope of the titration curve within this zone of color change. It follows that, when the end point is sharp, the slightest error in titration makes an enormous difference in the hydrogen ion concentration of the solution. A medium in which a sharp end point is found, is therefore, in general, not desirable. Such media have been automatically eliminated from general use.

In the following discussion, there will be occasion to assume a particular point on the titration curve as the end point of phenolphthalein. Since at $pH^+ = 8.50$ phenolphthalein is sufficiently pink to be distinctly noticeable in colored media, we shall assume this to be the so-called end point.

There is a further assumption involved; that is, that the indications of phenolphthalein in protein and salt solutions are comparable. There is good reason to believe that within certain narrow limits they are. This subject will be studied in detail in its relation to media and reported in due time.

[22] Color Standards and Color Nomenclature, 1912.
[23] Walpole, Jour. Chem. Soc., 1914, 105-106, p. 2501.
[24] Prideaux, Biochem. Jour., 1911, 6, p. 122.

THE BUFFER EFFECT OF MEDIA

The most important point shown by the titration curves is the buffer effect of the various media. The flatter the curve the greater is the buffer effect of the solution. This is shown clearly by a comparison of the titration curves of the 1 percent and the 5 percent peptone solutions (Chart 2). Both peptone solutions are initially very close to the point of absolute neutrality. On the addition of either acid or alkali, the hydrogen ion concentration of the 5 percent peptone solution changes much less than that of the 1 percent peptone solution, because of the higher content of substances which act as buffers (see under "Buffer" Effects). To bring the 5 percent peptone soluton down to $pH^+ = 8.50$ requires 2.5 c.c. n/10 NaOH to each 10 c.c., while to bring the 1 percent peptone solution to the same point requires only 0.5 c.c. n/10 NaOH to each 10 c.c. If $pH^+ = 8.50$ were assumed as the end point with phenolphthalein, the two solutions would have the "reactions" +25 and +5, Fuller's scale.[1] If, now, we should "correct" these solutions each to +10 Fuller's scale, the one would require the addition of 25—10 = 15 c.c. normal NaOH to each 1000 c.c., while the other would require the addition of 5 c.c. N *acid* to each 1000 c.c. After making these corrections, we should find the pH^+ value of the 5 percent peptone solution to be 7.80, while that of the 1 percent peptone solution would be 5.00.

These two solutions, then, both practically at absolute neutrality to begin with, have been "corrected" to the same "reaction;" yet the hydrogen ion concentration of the one has been left at a very favorable point for bacterial action, while the hydrogen ion concentration of the other has been raised almost to the limit of growth of Bacillus coli, and certainly far above the limit for numerous other organisms.

There occur in the literature some very striking instances of serious errors caused by neglect of such considerations.

It is true however that Dunham's solution, which is practically identical with the 1 percent peptone solution, is seldom or never "corrected." In text books are to be found elaborate directions for the "correction" of "reactions" and emphasis on its importance. Yet there is a significant absence of any direction for correcting Dunham's solution, or sometimes the specific direction that it should not be "adjusted." Experience seems to have dictated what theory could have predicted.

The comparison of the 1 percent and 5 percent peptone solutions furnishes what is perhaps an extreme case of the futility of titration methods of adjusting reactions. Cases less extreme, but of greater practical importance, are to be found in the comparison of media made with beef infusions and meat extract. In Chart 3 are the titration curves of such media, all containing 1 percent Witte peptone. One contains 0.3 percent Liebig's extract and another contains 0.5 percent Liebig's extract. The "0 C. infusion" medium was made up according to the directions of the American Public Health Association.[21] The

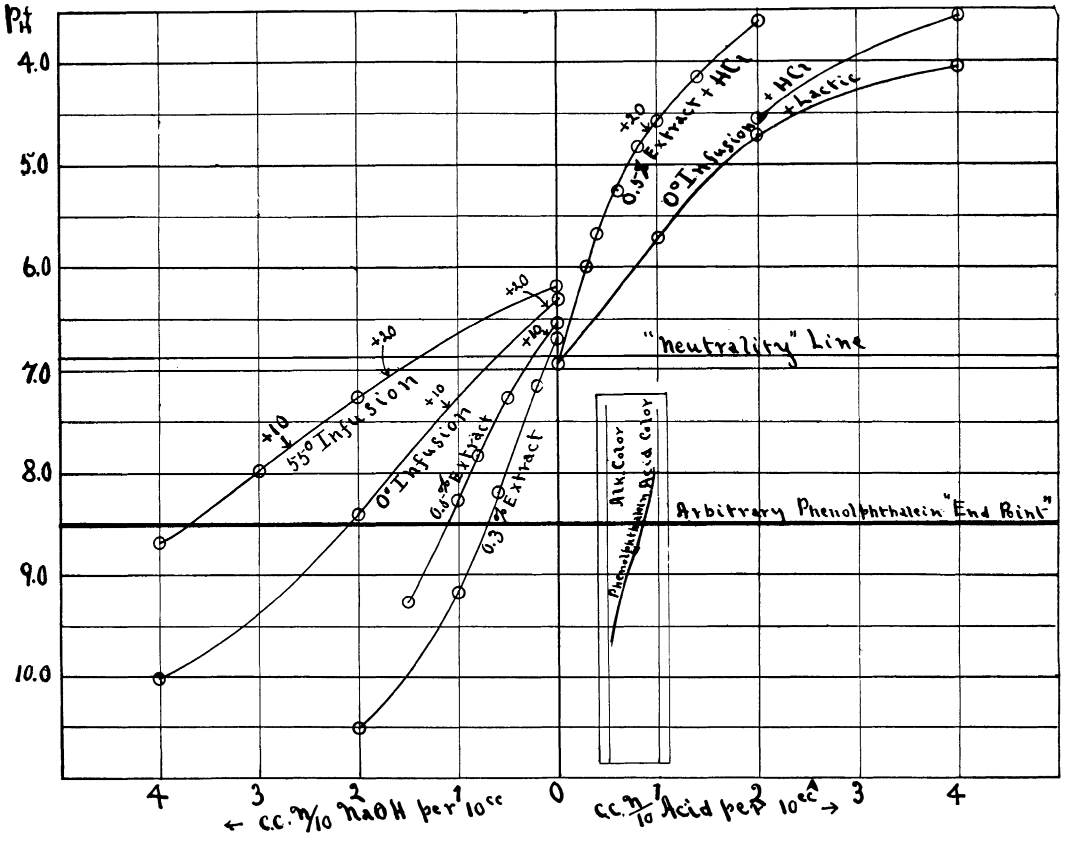

Chart 3.—Titration curves of infusion and meat extract broths.

"55 C. infusion" medium was made up according to Smith.[25] The titrations with NaOH were made before sterilization.

One of the most striking facts shown by these curves is the difference in the buffer content of the beef infusion made at 0 C. and that made at 55 C. This recalls the work of Gage and Adams,[26] who noted remarkable variation in the composition of infusions.

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[25] Bacteria in Relation to Plant Diseases, 1905.

Considering $pH^+ = 8.50$ as the end point with phenolphthalein and reckoning from where the curves cross the 8.50 line, we point off the +10 and +20 points (Fuller's scale). The location of these points shows again the futility of titration methods of adjusting reactions.

To show the comparative buffer effects of the 0 C. infusion and the 0.5 percent "extract" media when titrated with acid, the two media were brought to the same point, approximately neutral, and titrated with n/10 HCl and n/10 lactic acid. The difference in these two media in their "buffering" of added acid is of profound importance when such media are used to test the acid fermentation of sugars. If the same organism stops activity at approximately the same hydrogen ion concentration in each medium, the titratable acidity will vary considerably. Consequently, the titratable acidity furnishes no constant. It will vary, as Miss Broadhurst[27] has shown that it does, with the composition of the medium. Unless the composition is identical in every laboratory, no comparable data are obtainable. Comparable data are obtainable on another basis, as will be shown in other papers.

TITRATION CURVES OF MEDIA CONTAINING ADDED PHOSPHATES

In view of the fact that phosphates are extensively used in culture media, it may be useful to study a set of curves showing the effect of different phosphate mixtures upon a 1 percent peptone solution. Such curves are shown in Chart 4. These solutions were all titrated with lactic acid to give an approximate idea of the course which the hydrogen ion concentration would run during an acid fermentation of added sugar. In another paper, it will be shown that Bacillus coli, in accordance with the observations of Michaelis and Marcora,[4] brings these media up to approximately the same hydrogen ion concentration. A constant is thereby furnished. Titrations, on the other hand, would furnish a confused mass of data impossible to correlate on the old basis.

TITRATION CURVE OF MILK

It may be interesting to note also the titration curve of milk. In Chart 5 are given the titration curves of two samples of fresh, whole milk, titrated with NaOH, and of two other samples, titrated with lactic

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26. Jour. Infect. Dis., 1904, 1, p. 358.
27. Jour. Infect. Dis., 1913, 13, p. 404.

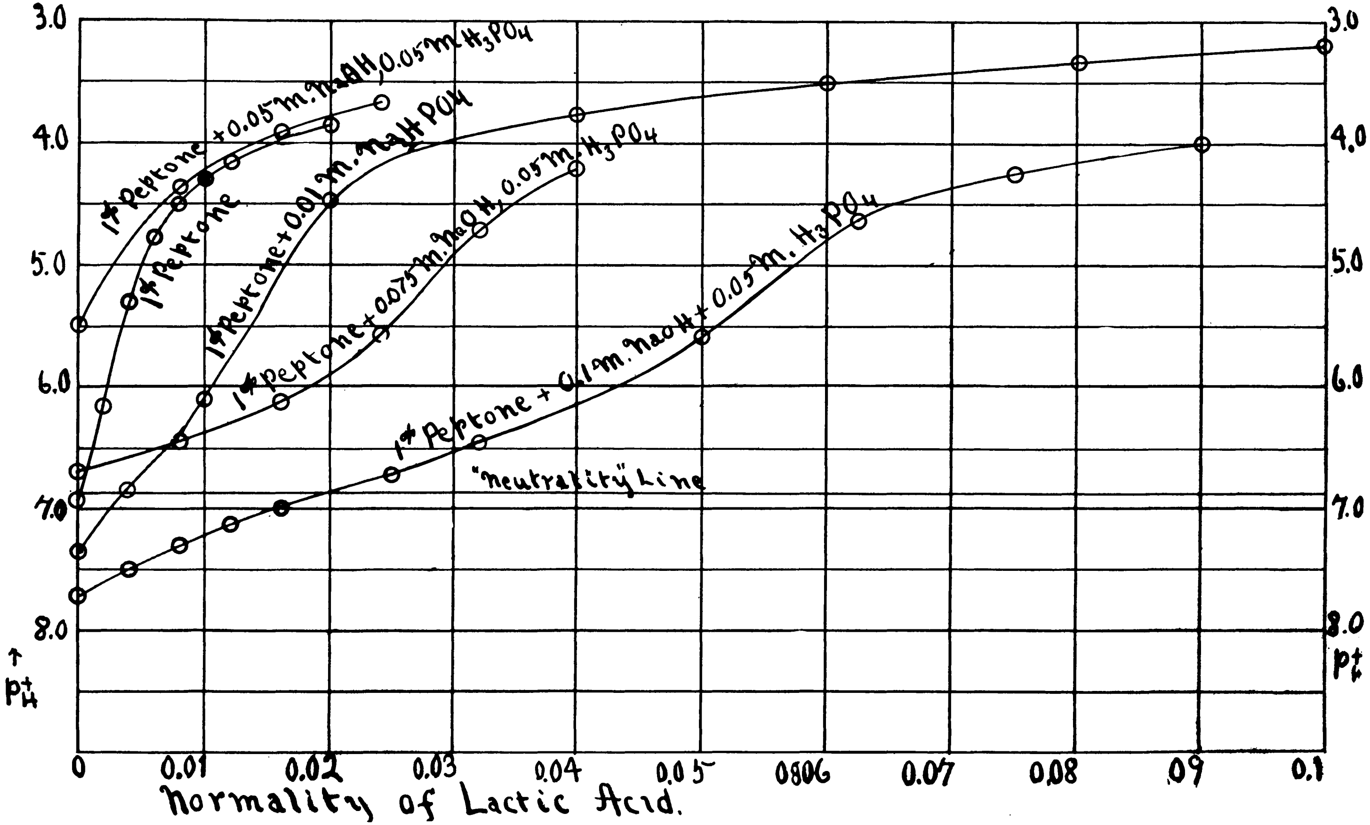

Chart 4.—Titration curves of 1 percent peptone solutions containing various mixtures of primary and secondary phosphates.

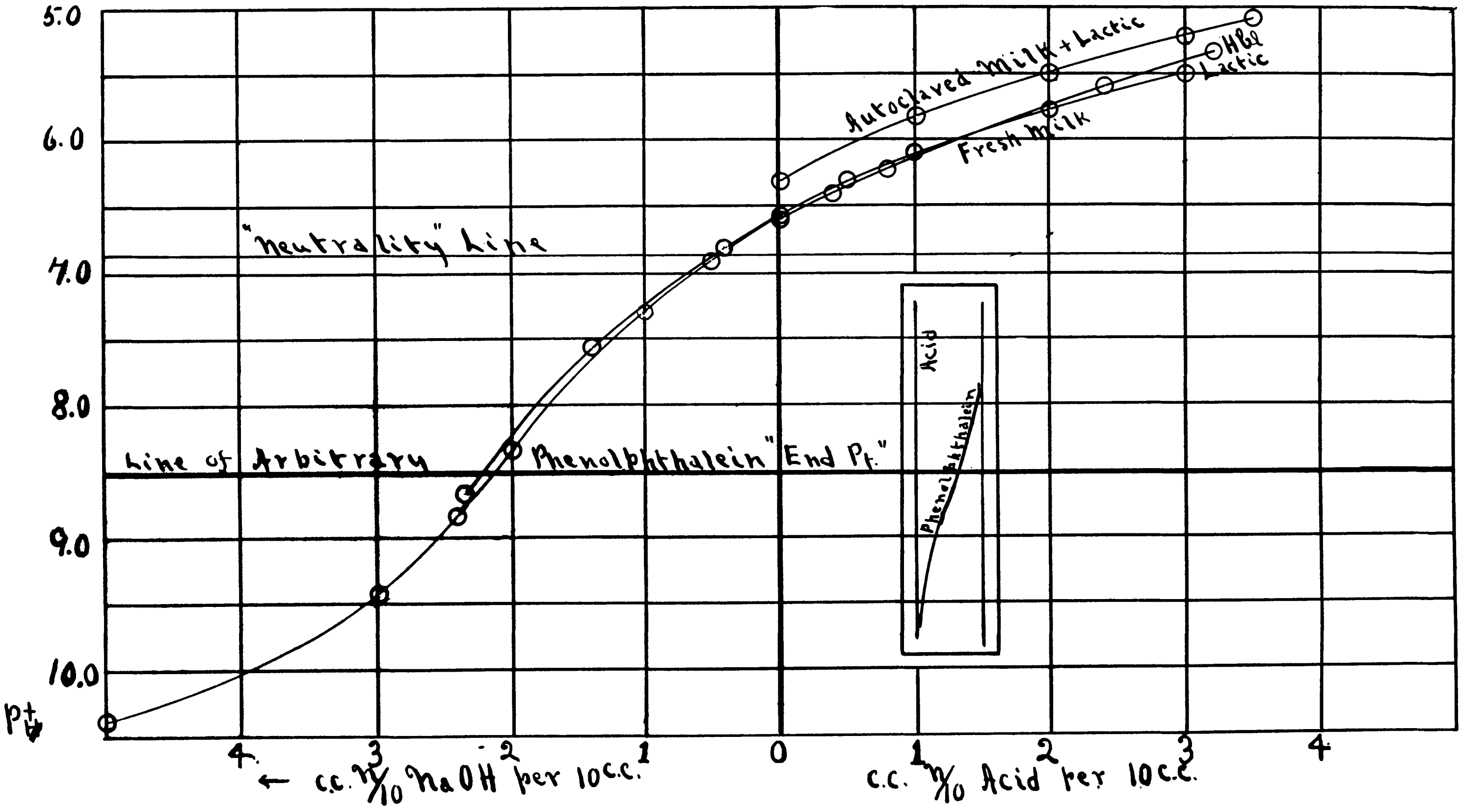

Chart 5.—Titration curves of fresh and heated cow's milk.

acid in one case and with hydrochloric acid in another. A portion of the sample which was titrated with lactic acid was titrated after heating. The heating raised the initial hydrogen ion concentration of the milk and so the titration curve of the heated sample lies above but parallel to that of the unheated portion. Titration curves of certain "modified" milks and human milk have been published in a previous paper.[28]

TITRATION CURVES OF SOLID MEDIA

Media containing agar can not be manipulated in the hydrogen-electrode apparatus used in these studies. It is not necessary to do so however, for the buffer effect of the agar itself or of its impurities, is probably very low, and consequently agar media have approximately the same titration curves as equivalent solutions of the other constituents. That the agar itself has the properties of a "buffer" to only a slight extent seems evident from the fact that 10 c.c. of a 1.5 percent agar solution required only one drop of n/10 NaOH to make it distinctly alkaline to phenolphthalein, and only one drop of n/10 H_2SO_4 to make it distinctly acid to methyl red.

Gelatin, on the other hand, may be kept sufficiently liquid at 30 C. to be manipulated. In Chart 6 are shown the titration curves of a medium consisting of 10 percent gelatin, 1 percent Witte peptone, 0.5 percent Liebig's extract. This mixture was first titrated with NaOH before "adjustment." It will be seen that the gelatin was considerably acid, for the initial pH^+ of the media was 5.6, while the initial pH^+ of the same media without gelatin was 6.55 (Chart 3). The hydrogen ion concentration was then adjusted to $pH^+ = 7.0$ and the medium sterilized. The hydrogen ion concentration rose during sterilization to $pH^+ = 6.75$. The titrations then gave the curve marked "adjusted and sterilized." It will be noticed that at $pH^+ = 6.75$ this medium was +12.5 Fuller's scale. A medium of the same composition made in another laboratory and adjusted by the ordinary procedure of dilution and heating during titration, was supposed to have a "reaction" of +10 Fuller's scale. As the curve marked "From laboratory C" shows, this medium was actually only +5 Fuller's scale, or 0.5 percent. The cause of this discrepancy will be shown later.

If, now, we compare the curves of the 10 percent gelatin, 1 percent peptone, 0.5 percent Liebig's extract medium (Chart 6) with that of the same medium without the gelatin (Chart 3), it will be seen that

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[28] Jour. Med. Research, 1915, 31, p. 431.

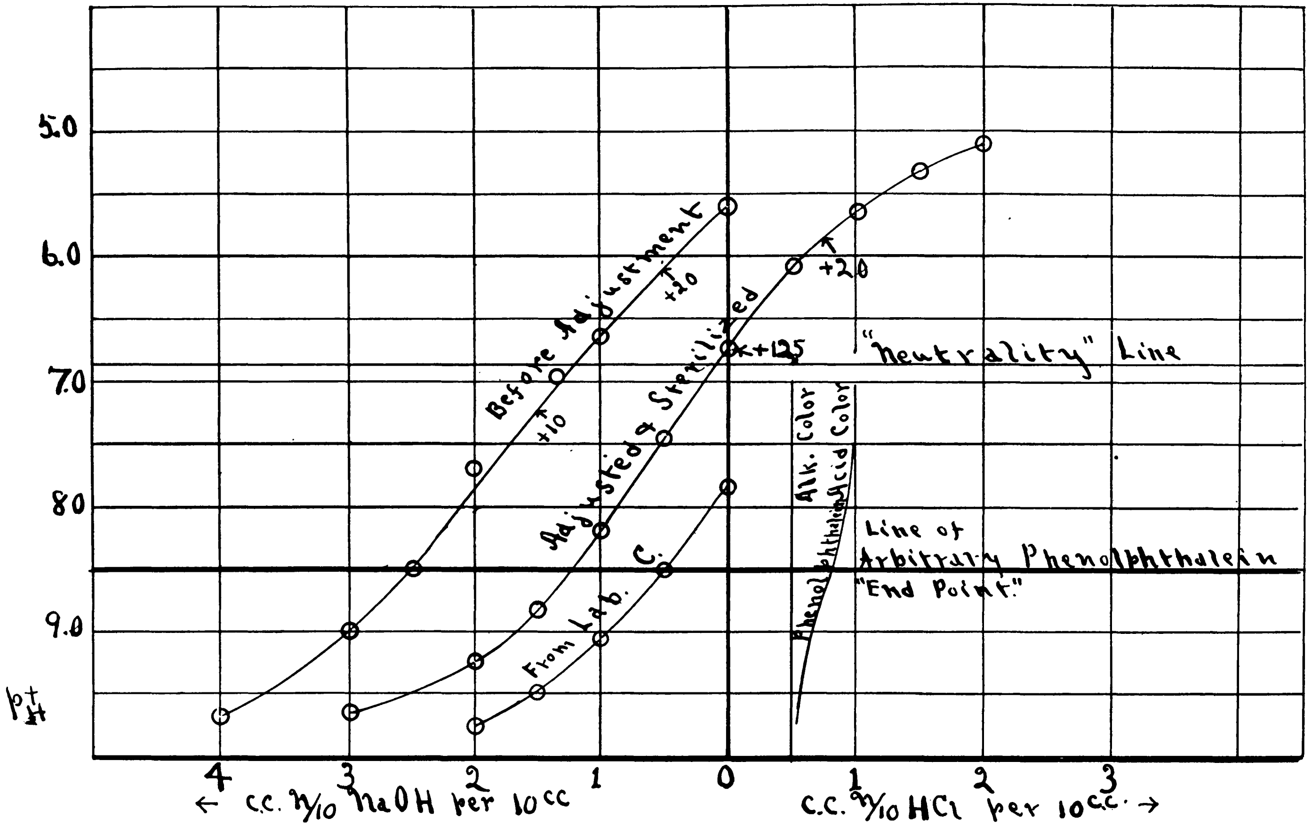

Chart 6.—Titration curves of a medium containing 10 percent gelatin, 0.5 percent Liebig's meat extract, and 1 percent Witte peptone.

the gelatin has increased the buffer effect appreciably, but that the gelatin by reason of its high molecular weight and few terminal carboxyl and animo groups is a much less effective buffer than the same weight of "peptone."

THE EFFECT OF HYDROGEN ION CONCENTRATION ON THE COMPOSITION OF MEDIA

Much of the attention given to reactions has been concentrated upon the reaction of meat-infusion media.

It is a well-recognized fact that many proteins exist in solution only within certain narrow limits of hydrogen ion concentration. Consequently, whatever the other factors may be which influence the composition of a complex mixture like a meat infusion, the hydrogen ion concentration is important. Even in a simpler solution, such as that of Witte peptone, we may observe the precipitation of various bodies as the hydrogen ion concentration is changed by the addition of acids, alkalies, and phosphates, or other acid or alkaline salts. In the case of meat infusions the situation is more complex, for there the slightest change in the hydrogen ion concentration is often sufficient to induce a heavy precipitate. In one instance, a change from $pH^+ = 6.33$ to $pH^+ = 6.95$ was found to cause a heavy flocculation. It is therefore evident that in dealing with such a medium it will make a considerable difference whether the medium is first brought to the pink reaction of phenolphthalein and then "adjusted" from this point (Timpe's method), or whether the adjustment is made by introducing only a fraction of the alkali necessary to reach the phenolphthalein point (Fuller's method).

Frequently the precipitations induced by a change in hydrogen ion concentration are not accelerated sufficiently to become very apparent until the medium is reheated. Then there occur changes also in the hydrogen ion concentration, which we shall consider next.

THE EFFECT OF HEAT UPON THE REACTION OF MEDIA

It is well known that media generally show an increase in titratable acidity after sterilization (Hesse[29]). It may be interesting to note first the effect of heat upon the hydrogen ion concentration of media in which very little precipitation occurs. Some data are shown in Table 2. The changes noted in the carbohydrate media are largely due to oxidations of the sugar, which are controlled in rate both by the temperature and the hydrogen ion concentration of the solution (Henderson[30]); also to the formation of "Melanins" (Mailliard[31]).

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[29] Ztschr. f. Hyg., 1904, 46, p. 1.

A somewhat different phenomenon occurs upon heating a mixture like meat infusion. Here constituents are precipitated and new equilibria involving the hydrogen ions are established.[32]

TABLE 2

pH^+ Values of Unsterilized and Sterilized Media Determined by Electrometric Measurements at 30 C.

Medium	Method of Sterilization	pH^+ Before	pH^+ After	Difference
1% Witte peptone	Autoclave	6.88	6.87	0.01
1% Witte peptone, 2% lactose	Autoclave Arnold	6.71 6.65	6.35 6.50	0.36 0.15
1% Witte peptone, 2% lactose, 0.050 MH_3PO_4, 0.050 NNaOH.	Autoclave Arnold	5.53 5.63	5.23 5.51	0.30 0.12
1% Witte peptone, 2% lactose, 0.050 MH_3PO_4, 0.075 NNaOH.	Autoclave Arnold	6.71 6.65	6.35 6.50	0.36 0.15
1% Witte peptone, 2% lactose, 0.050 MH_3PO_4, 0.100 NNaOH.	Autoclave Arnold	7.75 7.72	— 7.42	— 0.30
1% Witte peptone, 1% dextrose, 0.5% K_2HPO_4	Arnold	7.43	7.23	0.20
5% Witte peptone, 2% dextrose	Arnold	6.75	6.63	0.12
5% Witte peptone, 2% dextrose, 0.05 MH_3PO_4, 0.050 NNaOH.	Arnold	6.55	6.46	0.09
5% Witte peptone, 1% dextrose	Arnold	6.83	6.68	0.15
5% Witte peptone, 1% dextrose, 0.05 MH_3PO_4, 0.050 NNaOH.	Arnold	5.87	5.57	0.30
5% Witte peptone, 1% dextrose, 0.05 MH_3PO_4, 0.075 NNaOH.	Arnold	6.55	6.40	0.15
5% Witte peptone, 1% dextrose, 0.05 MH_3PO_4, 0.100 NNaOH.	Arnold	7.19	6.94	0.25
Skimmed milk	Autoclave	6.57	6.31	0.26
Skimmed milk	Autoclave	6.58	6.14	0.44

An infusion medium containing ½ percent K_2HPO_4 brought to $pH^+ = 7.19$ before sterilization changed to 6.70 after sterilization. Another portion brought to 8.52 changed to 7.72 after sterilization. In each case the change in hydrogen ion concentration was accompanied by a heavy precipitation.

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[30] Jour. Biol. Chem., 1911, 10, p. 3.

[31] Compt. rend. Acad. d. sc., Paris, 1912, 154, p. 66.

[32] Biochem. Ztschr., 1912, 44, p. 157.

Deeleman[33] recognized that the titratable acidity changes upon sterilizing media and therefore corrected his media by adding sterile acid or alkali after the medium was sterilized.

Increase in temperature also accelerates the solution of glass. In certain instances this may become serious, especially when synthetic media, poor in "buffers," are sterilized in new vessels of "soft" glass. In the majority of cases, however, this source of error is far outweighed and does not merit the emphasis placed upon it by W. Hesse[34] and his son.[29]

THE FALLACY OF TITRATING HOT MEDIA

Quite aside from any chemical change produced upon heating a medium, an elevation of temperature is accompanied by a change in the dissociation of the components of the medium. The increase in the dissociation of water is enormous. There may be an alteration also in the equilibrium of the tautometric forms of the indicator used. The effect of all these influences is to shift the titer. In certain simple cases, it may be possible to establish a precise relation between the titer of a hot solution and that of a cold solution (Bjerrum[11]), but for solutions as complex and variable as culture media it is impossible to do so. Redfield[35] has shown the great variations which may be procured by titrating peptone solutions at different temperatures. It is needless to add that, since titrations in the last analysis are based upon the attainment of a certain hydrogen ion concentration as shown by the tint of an indicator, the titration of a medium at 90-100 C. furnishes data of no exact significance at ordinary incubation temperatures. There is no procedure which deserves severer criticism than the titration of hot media. Aside from the fact that it is irrational, it has ruined the availability of an enormous body of data to be found in literature. Unless the exact procedure is given, one does not know the meaning of a +10 or a +15 reaction. But, even tho it were known, if the determinations were made by hot titrations, it would be impossible, except after laborious research, to translate these values by means of titration curves into hydrogen ion concentrations.

As an illustration, a 10 percent gelatin, 1 percent peptone, 0.5 percent meat extract medium was procured from a laboratory where the titrations were made after dilution and while the solutions were hot. This medium was reported to have a titratable acidity of +10

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[33] Arb. a. d. k. Gsndhtsamte, 1897, 13, p. 374.

[34] Ztschr. f. Hyg., 1893, 15, p. 183.

[35] A Study of Hydrogen Sulphide Production by Bacteria, Cornell Thesis, 1912.

Fuller's scale before sterilization. After sterilization, it should have been higher. Careful determinations with the hydrogen electrode and the plotting of a "titrative curve" (Chart 6) showed the acidity titratable to $pH^+ = 8.50$ to be +5.0. A careful titration with NaOH of the undiluted medium at room temperature and in the presence of phenolphthalein gave +6.0. Boiled in a vessel with a Bunsen valve, cooled, and titrated at room temperature, it gave +6.0. A similar titration of the undiluted medium near its boiling point gave +10. Five c.c. diluted to 50 c.c. with conductivity water and titrated hot gave +19. According to "official" methods, +19 should be the "true reaction." According to procedures sometimes used, +6 is the "true reaction." According to the temperature used and the end point assumed, the "true reaction" can be said to be anywhere between +4 and +20.

From the same laboratory, other batches of this medium were obtained, all of which were supposed to have been +10 before sterilization and all of which should have been higher than +10 after sterilization. By accurate determinations with the hydrogen electrode, they were found to be +7, +5, +8, +9, +2, +9, at 30 C. when reckoned from $pH^+ = 8.50$ as the "end point" of phenolphthalein.

THE INADEQUACY OF THE TITRIMETRIC METHOD

In the previous pages, it has been shown that: (1) Since the titration curves of culture media do not fall precipitously through the zone of hydrogen ion concentrations within which phenolphthalein changes color, there is no true "end point" in the commonly accepted meaning of that term.

(2) If a precipitous drop in the curve were found, it would indicate that in this region, at least, a very slight error in the amount of alkali added would alter the hydrogen concentration enormously. Such a medium would be practically useless for the cultivation of bacteria sensitive to changes in hydrogen ion concentration.

(3) It is necessary to assume some tint of phenolphthalein as a standard if the titrimetric method is to be accurate. The present paper color standard has no physical significance for the problem at hand, while the tint assumed by a definite amount of phenolphthalein in a standard solution of known hydrogen ion concentration has a precise physical significance.

(4) The tint of phenolphthalein at $pH^+ = 8.50$ being assumed as a standard, it has been shown that, when media are "corrected" from

this point by the titrimetric method, the degrees (Fuller scale) correspond to very different hydrogen ion concentrations in various media.

(5) Altho different batches of the same medium might be brought to the same hydrogen ion concentration by accurate titrations, this can not be done if the temperature of titration is varied. Furthermore, the titer obtained while a medium is hot has no precise meaning when the datum is applied at incubation temperature.

In short, when a medium is titrated, all that is accomplished is to reach a more or less definite hydrogen ion concentration as determined by the color of an indicator. The amount of alkali required to obtain this hydrogen ion concentration depends upon the original hydrogen ion concentration of the medium and upon the buffer content of the medium. The addition of alkali short of the amount necessary to produce a given tint of phenolphthalein leaves the medium at a hydrogen ion concentration which is indeterminate, unless the titration curve is known.

THE COLORIMETRIC METHOD

It is quite evident that if a medium is to be adjusted to $pH^+ = 8.50$, this point can be obtained accurately by simply adding alkali until the color of the medium with phenolphthalein present matches the color of a standard solution of $pH^+ = 8.50$ with phenolphthalein present. If it is desired to bring the medium to some hydrogen ion concentration outside the range of phenolphthalein, another indicator useful within the desired range may be chosen. Next to phenolphthalein, the indicator most commonly used is litmus. Unfortunately litmus, according to Walpole[19] and others, can be used for this purpose only with a considerable error. But in view of the fact that a fair success has been attained in the cultivation of bacteria in media adjusted to variable hydrogen ion concentrations by the titrimetric method, it is doubtless true that the error in adjusting to some tint of litmus is of no great consequence.

A complete set of indicators suitable for the accurate adjustment of culture media, together with a study of the difficulties involved in the procedure, are being investigated in this laboratory.

GENERAL CONSIDERATIONS

It is evident that media, as they are corrected in practice, must vary considerably in their hydrogen ion concentrations. Yet a considerable degree of success in cultivating bacteria seems to have been attained. The question then arises whether the reaction of a medium is so important as frequent assertions would lead one to believe. In attempting to answer this question, one may say at once that the initial hydrogen ion concentration, if it varies within certain limits, is not at all important so far as the mere growth of certain organisms is concerned. In this laboratory, we have had occasion to grow Bacillus coli and certain streptococci in media with pH^+ values varying from 5.5 to 9.0 and have obtained good growths in all. With favorable food supplies, it is probable that many other species can flourish within the same limits. In such cases, the hydrogen ion concentration of the medium makes itself felt not at all in an entire suppression of activity, but in the rates of different fermentations. We then find, as Blumenthal[36] did as early as 1895, that the acidity or alkalinity of the medium has an appreciable influence on the relative proportions of the end products of fermentation or putrefaction.

On the other hand, certain species, notably certain pathogens, are reported to be very sensitive to slight changes in titratable reaction. When we are considering the effect upon such sensitive organisms, we must take into consideration the medium as well as the so-called titratable acidity. A titratable acidity of 10 in one medium may furnish an optimal hydrogen ion concentration, while 10 in another medium might be unendurable to any organism. It is certainly significant that the beef infusion media, corrected by customary methods, have hydrogen ion concentrations varying between the neutral point of water and that of blood, and that the buffer effect of infusion media is so great that no great change is made in the hydrogen ion concentration by a considerable variation in the titratable acidity. From such considerations we may conclude that in certain instances the influence of reaction has been overestimated because the experiments were conducted in buffer-poor media; while in other instances the effect of reaction has been underestimated because considerable variations in added acid or alkali had little influence on the hydrogen ion concentration of richly buffered media.

In other cases in which the hydrogen ions have been considered, their influence has not been demonstrated or disproved, because their concentration has been calculated on the basis of the dissociation of acids in pure water, and no account has been taken of the "buffers." Some of the work on yeasts seems to have suffered from this oversight.

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[36] Ztschr. f. klin. Med., 1895, 28, p. 223.

An instance in which considerable variations in hydrogen ion concentration would probably have little effect is in making counts of the bacteria found in milk. As a rule, the initial spoilage of milk is accompanied with an acid fermentation, and the great majority of the organisms found in milk during the early stages of the spoiling are more or less acidophilic and capable of flourishing in a considerable range of hydrogen ion concentration. It is therefore almost certain that if the food supply in a meat extract broth were comparable to that in a meat infusion broth, for the organisms in question, the two media would not differ greatly in value even if the initial titratable acidity in both were the same and, consequently, the hydrogen ion concentration of one were higher than that of the other. If, however, the flora of a milk is to be investigated in detail, it is possible that the initial reaction of the media may have an appreciable influence upon the relative number of some forms which appear upon a plate; for here relative rates of growth are of chief importance. A striking instance of this is given by Rogers and Ayers.[37]

The possibilities are that for the cultivation of the majority of bacteria it will do quite as well to leave many media unadjusted and thereby insure a more constant composition.

On the other hand, if many organisms are as sensitive as innumerable statements in the literature imply, an entirely new basis of adjusting reactions will doubtless have to be devised before comparable data can be obtained.

It certainly is suggestive to remember that the blood contains a powerful mechanism for the regulation of its hydrogen ion concentration (Henderson[38]). It has been shown that small alterations in this concentration are attended by serious consequences to the cells and to the enzymes which find blood their natural medium. It was a similar consideration which led Henderson and Webster[39] to propose their phosphate mixture for the regulation of the hydrogen ion concentration of bacterial cultures.

Likewise, Sørensen and Palitzsch[40] have shown the remarkable constancy of the hydrogen ion concentration of the oceans, and Henderson[41] has vividly described the biologic importance of the carbonate equilibrium which makes this constancy possible.

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[37] U. S. Dept. Agriculture, 1910, Circular 153.

[38] Ergebn. d. Physiol., 1909, 8, p. 254.

[39] Jour. Med. Research, 1907, 16, p. 1.

[40] Biochem. Ztschr., 1910, 24, p. 387.

[41] The Fitness of the Environment. An Inquiry into the Biological Significance of the Properties of Matter, 1913.

Bacteria in the great majority of their natural habitats find one or more buffers which tend to neutralize the acid or basic products of their metabolism and thus permit an extension of bacterial action. When, therefore, bacteria are transplanted to artificial media, they should be provided there, not only with proper food, but with buffers to protect them from the toxic effect of the acid or basic products of their metabolism. Not only must this be done, but, if the maximal rate of activity is to be attained, the organisms must find the hydrogen ion concentration of the medium suitable for the optimal activity of their enzymes and consistent with the stability of their colloidal structures. In many instances a considerable variation in hydrogen ion concentration may be tolerated, but only a narrow range may be suitable for optimal rates of these processes, upon which many commercial and academic questions depend.

CHART REPRESENTATION OF PHYSIOLOGICALLY IMPORTANT HYDROGEN ION CONCENTRATIONS

The data already presented will have a clearer signficance if we have before us a graphic representation of the hydrogen ion concentrations of certain biologic fluids and the zones of enzyme action. Such a representation has been attempted in Chart 7. This has been compiled for the most part from the data given in Michaelis'[4] book. In representing the hydrogen ion concentration (as pH^+ values) of blood, for instance, it must not be understood that blood is always at the point indicated. It varies slightly, cows' milk slightly, muscle juice in proportion to the degree of autolysis, and feces with the diet. The extent of the variations will be found in original papers. Likewise, the representations of the zones of enzyme action are approximations, designed only to give a general idea.

This chart is similar to, but much less complete than Walpole's.[20] In one respect, however, it is believed to have an advantage. Walpole gives only the optimal points for various enzymes and leaves the reader rather confused as to the zone of hydrogen ion concentration throughout which any particular enzyme can operate. In the present chart, the optimum for trypsin, for instance, is shown at $pH^+ = 8.00$, but the enzyme still preserves about 50 percent of its activity at $pH^+ = 6.6$, and reaches minimal activity only at about $pH^+ = 5.0$.

It is important to note the breadth of these zones, for if bacterial enzymes are similar to the better known enzymes, they are operative over a considerable range of hydrogen ion concentration. At the same time, it is very important to recognize that altho an enzyme may be active outside its optimal range, its rate of action may be enormously diminished. In symbiotic processes, especially, rates are all important.

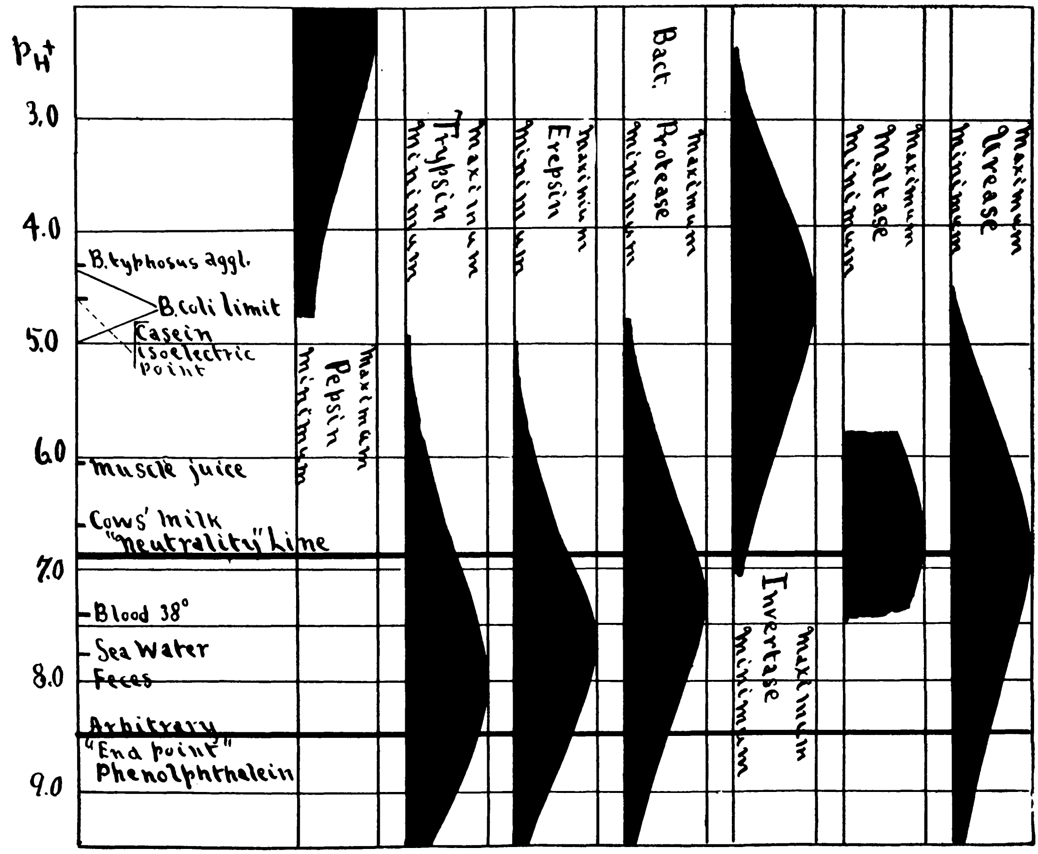

Chart 7.—Representation of hydrogen ion concentrations of physiologic importance, and of the approximate range and degree of activity of several enzymes.

SUMMARY

The principles of titration have been briefly reviewed. The hydrogen ion concentrations of various culture media before and after the addition of successive quantities of alkali and acid have been determined by means of the hydrogen electrode. From the data so obtained, "titration curves" have been constructed which show (1) the amount of acid or alkali which it is necessary to add to certain media in order to reach a given hydrogen ion concentration such as would be indicated, for instance, by a certain tint of phenolphthalein; (2) the buffer effect of various media; (3) the hydrogen ion concentrations of different media adjusted to various degrees of reaction (Fuller's scale). It is shown that adjustment of different media to the same degree of reaction (Fuller's scale) may result in media having widely different hydrogen ion concentration.

The titrimetric method is further shown to be vitiated by the custom of titrating media while hot.

The fallacies of the titrimetric method of adjusting culture media have been shown to point to the advisability of its abandonment and to the substitution of a colorimetric method.

The influence of hydrogen ion concentrations upon the composition of complex media has been touched upon. Some data have been presented showing the influence upon the hydrogen ion concentration of oxidations of carbohydrates and precipitation of proteins when accelerated by high temperatures.

The significance of the proper adjustment of the hydrogen ion concentration of media has been discussed and certain points illustrated by means of a chart representation of hydrogen ion concentrations of physiologic importance.